ELSEVIER

Journal of Immunological Methods 245 (2000) 147–152

JIM
Journal of
Immunological Methods

www.elsevier.nl/locate/jim

Protocol

A simple and rapid flow cytometric method for detection of porcine cell surface markers

Thomas J. Stabel*, Steven R. Bolin, Bruce A. Pesch, Theresa E. Rahner

United States Department of Agriculture, Agricultural Research Service, National Animal Disease Center, 2300 Dayton Road, Ames, IA 50010, USA

Received 14 March 2000; received in revised form 14 July 2000; accepted 30 July 2000

Abstract

The objective of this study was to develop a rapid and reliable method for flow cytometric analysis of porcine whole blood cells. Fifty-microliters of heparin- or EDTA-treated whole blood was added to wells of a round-bottom 96-well microtitration plate. Each well contained 10 μl of an appropriate dilution of four different antibodies (40 μl total; two primary monoclonal antibodies and two fluorescent-labeled secondary antibodies). For convenience, the antibody mixture could be added to plates 1–2 days prior to assay and stored at 4°C. Once whole blood was added to wells, plates were mixed gently, placed in a sealed bag and incubated in the dark at room temperature for 20 min. Contents of wells were then transferred to polystyrene tubes containing 2 ml of 1.5% formalin in distilled water and mixed gently. Cells were fixed for a minimum of 30 min and then stored in the dark at 4°C until analysis by flow cytometry. Analysis of cell samples may be done up to 3 days after fixation. Results indicate that the percentages of Class I, Class II, CD3, CD8, CD4, CD45, monocyte, gamma-delta T-cell populations, and total number of granulocytes identified using this method were comparable to standard values or to values obtained following separation of white blood cells from red blood cells. The percentage of labeled B-cells was lower than standard values. Total assay time from receipt of blood to acquisition of data by flow cytometry required less than 2 h. This modified assay was shown to be simple, reliable, and useful for screening large numbers of porcine samples in a minimal period of time.

Keywords: Flow cytometry; Porcine; Cell surface markers

1. Description

Flow cytometry provides a rapid method to analyze normal and abnormal cell populations using a small sample volume and the results obtained accurately reflect the in vivo status of the populations (Boeker et al., 1999; van Eeden et al., 1999). Most procedures for labeling of cell surface markers require considerable manipulation of the cells and involve numerous time consuming wash steps (Fuertes et al., 1999). Recently, a simplified whole blood flow cytometric assay was reported for bovine leukocyte markers; however, multiple wash steps were still required (Pesch et al., 1997). Another recent report describes a method for analysis of human whole blood that requires only one wash step after lysis of red blood cells (McCloskey et al., 1997). The procedure described here uses whole

*Corresponding author. Tel.: +1-515-663-7292; fax: +1-515-663-7458.

E-mail address: tstabel@nadc.ars.usda.gov (T.J. Stabel).

0022-1759/00/$ – see front matter
PII: S0022-1759(00)00289-1

blood, instead of partially purified cells, and allows simultaneous addition into the well of a 96-well microtitration plate all the reagents required for two-color analysis of porcine leucocytes. At the final step, cells are removed from the microtitration wells and added to standard 12×75 mm polystyrene test tubes for simultaneous lysing of red blood cells and fixation. This procedure requires no wash steps.

2. Time required

1. Preparation of primary and secondary antibody stock dilutions (in advance): 30 min.
2. Labeling of tubes and addition of 1.5% formalin (in advance): 30 min for one plate.
3. Preparation of antibody series in microtitration plate (in advance): 30 min for one plate.
4. Addition of whole blood to wells of plate: 10 min for one plate.
5. Incubation of cells at room temperature in the dark: 20 min.
6. Transfer of contents from each well to formalin tubes: 45 min for one plate.
7. Fixation of cells: 30 min.
8. Analysis of cells by flow cytometry: 1.5 h for one plate.

3. Materials

1. Preparation of fluorescence buffer (FB)

- 0.01 M sodium phosphate buffered 0.9% saline (PBS; Sigma).
- Heat-inactivated fetal bovine serum (FBS; Hyclone) (1%); to heat inactivate serum, heat at 56°C for 30 min; store at −20°C.
- Sodium azide (0.05%) (Sigma).

2. Preparation of antibody solutions

- Class I MHC (≠PT85A; IgG_{2a}; Veterinary Medical Research and Development Inc., (VMRD)); dilute 1:60 in FB.
- Class II MHC (≠TH16B; IgG_{2a}; VMRD); dilute 1:60 in FB.
- CD3 (≠2B3C; IgG_{2b}; VMRD); dilute 1:30 in FB.
- CD8 (≠76-2-11; IgG_{2a}; VMRD); dilute 1:30 in FB.
- CD45 (≠74-9-3A1; IgM; VMRD); dilute 1:60 in FB.
- B cell (≠BB6-11C9; IgG_1; VMRD); dilute 1:60 in FB.
- Granulocyte/Monocyte (≠74-22-15; IgG_1; VMRD); dilute 1:60 in FB.
- Gamma/Delta T-cell (≠PGBL22A; IgG_1; VMRD); dilute 1:60 in FB.
- Granulocyte (≠PG68A; IgG_1; VMRD); dilute 1:30 in FB.
- CD4 (≠PT90A; IgG_{2a}; VMRD)); dilute 1:60 in FB.
- Anti-mouse IgG2a-FITC (≠1082-02; Southern Biotechnology Association (SBA)); dilute 1:40 in FB.
- Anti-mouse IgG1-FITC (≠1072-02; SBA); dilute 1:40 in FB.
- Anti-mouse IgG2b-PE (≠1092-09; SBA); dilute 1:40 in FB.
- Anti-mouse IgG1-PE (≠1072-09; SBA); dilute 1:40 in FB.
- Anti-mouse IgG2a-PE (≠1082-09; SBA); dilute 1:40 in FB.
- Anti-mouse IgM-PE (≠1022-09, SBA); dilute 1:40 in FB.

3. Preparation of 1.5% formalin

- 10% buffered formalin (Anatech, Ltd.); dilute to 1.5% with d(H_2O).

4. Blood collection

- Heparin or EDTA-vacutainer tubes (Becton–Dickinson).
- 20 gauge, 1.5 inch multiple sample needles (Becton–Dickinson).

5. Assay

- Round-bottom 96-well microtitration plates (Costar).
- Microplate sealers (Sealplate, Daigger).
- 12×75 mm polystyrene tubes (Falcon).

3.1. Special equipment

- Autoclave.
- Sterile autoclaved micropipette tips (200 μl, 1000 μl) (Bio-Rad).
- Pipettors (20 μl, 100 μl, 1000 μl) (Gilson).
- Multichannel pipette (20–200 μl) (Costar).
- Flow cytometer (Facscan, Becton–Dickinson).

4. Detailed procedure

4.1. Preparation of antibody plate(s)

1. In advance, prepare separate stocks of primary (7–33 μg/ml) and secondary (6–13 μg/ml) antibody solutions sufficient for the entire experiment. Dilute antibodies in fluorescence buffer (FB); stocks prepared in FB may be stored for several months in the dark at 4°C.
2. Add 10 μl/well of each primary and secondary monoclonal antibody into a round-bottom 96-well microtitration plate. For two-color analysis, add two different primary antibodies and two different fluorochrome-labeled secondary antibodies (40 μl total). Wells containing antibody may be prepared 1–2 days in advance, if covered with a plate sealer and stored in the dark at 4°C.

4.2. Collection of blood (porcine)

1. From the jugular vein or anterior vena cava, collect a full tube of porcine whole blood into a heparin- or EDTA-treated vacutainer tube (<1 ml is required for assay). Pigs were cared for in accordance with American Association for Accreditation of Laboratory Animal Care guidelines.

4.3. Cell labeling

1. To wells containing antibody, add 50 μl whole blood per well, mix gently, and incubate in a sealed bag in the dark at room temperature for 20 min.
2. After incubation, transfer contents of each well to

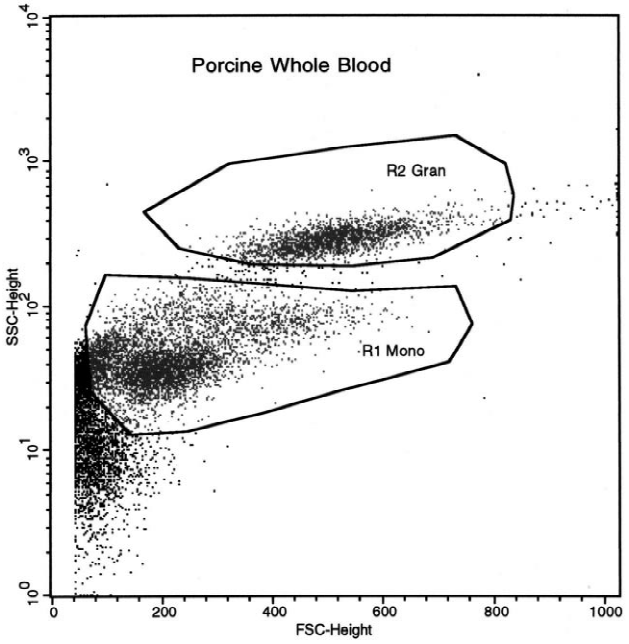

Fig. 1. Dotplot demonstrating forward light-scatter versus side light-scatter properties of porcine leukocytes after treatment in rapid whole blood two-color cytofluorometric assay. Mononuclear cells are included in the ''R1 Mono'' gate and granulocytes are included in the ''R2 Gran'' gate.

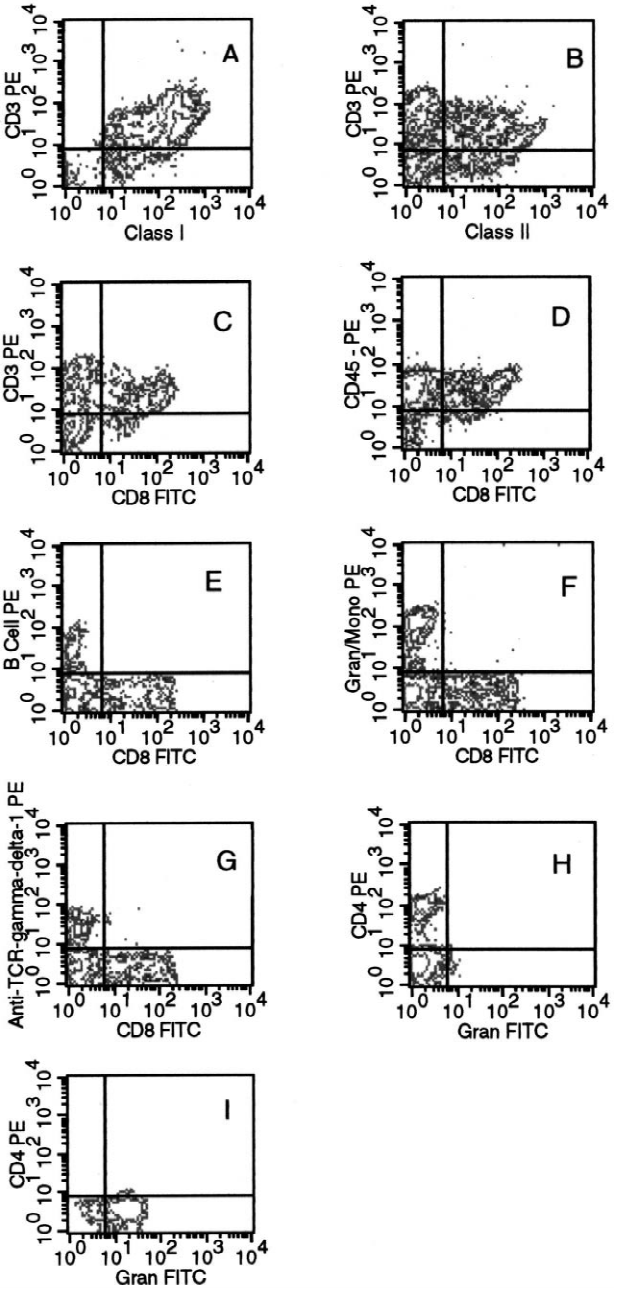

Fig. 2. Two-dimensional contour plots of porcine leukocytes using the rapid whole blood two-color cytofluorometric assay. Phycoerythrine (PE)- or fluorescein isothyocyanate (FITC)-conjugated monoclonal antibodies were used to label CD3 (A–C), CD4 (H), CD8 (C–G), MHC-Class I (A), MHC-Class II (B), CD45 (D), B (E), monocyte/macrophage (F), γδ T (G), and granulocyte (I) specific monoclonal antibody bound to cells.

12×75 mm polystyrene tubes containing 2 ml of 1.5% formalin diluted in water. Mix gently.
3. Fix cells for a minimum of 30 min and then store in the dark at 4°C until analysis by flow cytometry.

4.4. Flow cytometry (acquisition and analysis)

1. Double-stained cells are analyzed using a Becton–Dickinson FACScan flow cytometer, utilizing CellQuest software. Analysis gates are determined by backgating on two-dimensional forward-scatter×side-scatter histograms. Fluorescence statistics are determined from the gated populations using 50% log density contour plots and quadrant statistics.

5. Results

A representative dot plot and series of contour plots from a single pig (9 weeks-old) are shown in Figs. 1 and 2. Statistical results from Fig. 2 contour plots and plots from 12 additional pigs (data not shown) are presented in Table 1 as ''% Gated'' and ''Average % Gated'', respectively. Values represent the percentage of the gated mononuclear cells (R1 Mono) or the percentage of granulocytes (R2 Gran) in the total whole blood population. Isotype controls, included in initial stages of methods development, caused no significant non-specific binding of secondary antibody to cell populations (data not shown).

Table 1
% of porcine peripheral blood leukocytes positive for antigen using new flow cytometric analysis method

Cell surface marker	% Gated[a]	Average % gated[b]
CD3	54.68	66.21±0.99
Class I	96.67	95.18±0.37
Class II	43.29	29.14±0.38
CD8	26.27	24.26±0.48
CD45	79.08	74.63±1.77
B-cell	8.40	8.12±0.33
Mono	14.80	14.72±0.36
γ/δ	16.88	17.78±0.91
CD4	14.63	27.80±1.15
Gran	19.91 (% of total)	18.33±0.94

[a] Data from Figs. 1 and 2.
[b] Thirteen pigs were assayed on 7 different days over a period of 4 weeks. Data represents the mean±standard error of the mean.

6. Discussion

Percentages of Class I, Class II, CD3, CD8, CD4, CD45, monocytes, γ/δ T-cells, and total number of granulocytes were comparable to previously reported values for the same age group: 85–90% (Davis et al., 1987), 16–37% (Davis et al., 1987), 55–70% (personal communication, Joan Lunney), 25% (Zuckermann and Husmann, 1996), 20% (Zuckermann and Husmann), 86% (Pescovitz et al., 1984), 15% (Hernández et al., 1998), 16% (Boeker et al., 1999), 25–45% (standard range value; Veterinary Pathology Clinic, College of Veterinary Medicine, Iowa State University, Ames, IA), respectively. The percentage of labeled B-cells was lower than reported values (20%) (Shimizu et al., 1996). Total assay time from receipt of blood in the laboratory until acquisition of data by flow cytometry required less than 2 h. In our hands, this modified whole blood assay was simple, reproducible, and rapid when used to evaluate up to 15 samples of porcine blood for 10 cell surface markers (150 individual tubes).

References

Boeker, M., Pabst, R., Rothkotter, H.J., 1999. Quantification of B, T and Null lymphocyte subpopulations in the blood and lymphoid organs of the pig. Immunobiology 201, 74.

Davis, W.C., Marusic, S., Lewin, H.A., Splitter, G.A., Perryman, L.E., McGuire, T.C., Gorham, J.R., 1987. The development and analysis of species specific and cross reactive monoclonal antibodies to leukocyte differentiation antigens and antigens of the major histocompatibility complex for use in the study of the immune system in cattle and other species. Vet. Immunol. Immunopathol. 15, 337.

Fuertes, L.L., Doménech, N., Alvarez, B., Ezquerra, A., Domínguez, J., Castro, J.M., Alonso, F., 1999. Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection. Virus Res. 64, 33.

Hernández, J., Reyes-Leyva, J., Zenteno, R., Ramírez, H., Hernández-Jauregui, P., Zenteno, E., 1998. Immunity to porcine rubulavirus infection in adult swine. Vet. Immunol. Immunopathol. 64, 367.

McCloskey, T.W., Cavaliere, T., Bakshi, S., Harper, R., Fagin, J., Kohn, N., Pahwa, S., 1997. Immunophenotyping of T lympho-

cytes by three-color flow cytometry in healthy newborns, children, and adults. Clin. Immunol. Immunopathol. 84, 46.

Pesch, B., Rajaraman, V., Kehrli Jr., M.E., Harp, J.A., 1997. New flow cytometric techniques for the analysis of blood from large animals. First International Virtual Conference on Infectious Diseases of Animals (www.nadc.ars.usda.gov/virtconf). Poster #G00081.

Pescovitz, M.D., Lunney, J.K., Sachs, D.H., 1984. Preparation and characterization of monoclonal antibodies reactive with porcine PBL. J. Immunol. 133, 368.

Shimizu, M., Yamada, S., Kawashima, K., Ohashi, S., Shimizu, S., Ogawa, T., 1996. Changes of lymphocyte subpopulations in pigs infected with porcine reproductive and respiratory syndrome (PRRS) virus. Vet. Immunol. Immunopathol. 50, 19.

van Eeden, S.F., Klut, M.E., Walker, B.A.M., Hogg, J.C., 1999. The use of flow cytometry to measure neutrophil function. J. Immunol. Methods 232, 23.

Zuckermann, F.A., Husmann, R.J., 1996. Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T-cells. Immunology 87, 500.